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ImmunoChip Analysis Identifies Multiple Susceptibility Loci for Systemic Sclerosis

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In this study, 1,833 systemic sclerosis (SSc) cases and 3,466 controls were genotyped with the ImmunoChip array. Classical alleles, amino acid residues, and SNPs across the human leukocyte antigen (HLA) region were imputed and tested. These analyses resulted in a model composed of six polymorphic amino acid positions and seven SNPs that explained the observed significant associations in the region. In

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addition, a replication step comprising 4,017 SSc cases and 5,935 controls was carried out for several selected non-HLA variants, reaching a total of 5,850 cases and 9,401 controls of European ancestry. Following this strategy, we identified and validated three SSc risk loci, including *DNASE1L3* at 3p14, the *SCHIP1-IL12A* locus at 3q25, and *ATG5* at 6q21, as well as a suggested association of the *TREH-DDX6* locus at 11q23. The associations of several previously reported SSc risk loci were validated and further refined, and the observed peak of association in *PXK* was related to *DNASE1L3*. Our study has increased the number of known genetic associations with SSc, provided further insight into the pleiotropic effects of shared autoimmune risk factors, and highlighted the power of dense mapping for detecting previously overlooked susceptibility loci.

Introduction

Systemic sclerosis (SSc, scleroderma [MIM 181750]) is an autoimmune disease characterized by three main features: (1) fibrosis of the skin and internal organs, (2) a noninflammatory vasculopathy, and (3) autoantibody production.¹ It is a multiorgan system disease with considerable phenotypic heterogeneity, resulting in a broad spectrum of disease severity. From a clinical point of view, SSc is divided into limited cutaneous systemic sclerosis (lcSSc) or diffuse cutaneous systemic sclerosis (dcSSc).² From an immunological point of view, SSc is typically classified according to mutually exclusive and disease-specific autoantibodies (anticentromere antibodies [ACAs] and antitopoisomerase antibodies [ATAs]), which are found in approximately 50% of SSc cases.³

Several studies have established that SSc is a complex genetic disease with contributions from multiple genetic loci.^{4,5} The evidence supporting a genetic predisposition for the disease has revealed a major contribution from the major histocompatibility complex (MHC), as well as a number of other gene regions.^{4–6} In fact, genome-wide association studies (GWASs) have confirmed the MHC class II region as the most significant genetic region associated with SSc.^{7,8} Interestingly, a recent report restricted the MHC associations to the different autoantibody subsets.⁹ In addition, multiple non-MHC loci, such as *IRF5* (MIM 607218), *STAT4* (MIM 600558), *CD247* (MIM 186780), *TNIP1* (MIM 607714), *IRF8* (MIM 601565), *IL12RB2* (MIM 601642), *CSK* (MIM 124095), *KIAA0319L* (MIM 613535), *PXK* (MIM 611450), *JAZF1* (MIM 606246), *BLK* (MIM 191305), *ITGAM* (MIM 120980), and *TNFAIP3* (MIM 191163), have been associated with this condition at the genome-wide significance level ($p < 5 \times 10^{-8}$).^{7–13} Both GWASs and candidate-gene strategies have clearly identified SSc susceptibility factors involved in different components of the immune system (innate immune response, adaptive immune response, cytokines, cytokine receptors, etc.), as well as genes involved in pathways that might play a role in vascular damage and fibrotic processes.^{4–6} Despite the success of these approaches, innovative strategies are needed for dealing with the remaining unmapped SSc heritability.

Hence, the next step in the genetic dissection of complex autoimmune diseases (AIDs) is to identify the causal variants for the already established susceptibility loci, as well as to discover variants with lower penetrance with the use of larger cohorts. Furthermore, these goals need to be achieved with a cost-efficient strategy.^{14,15} To this

end, an international group of collaborators formed the Immunochip Consortium to develop and implement the Immunochip array, a custom SNP genotyping array that provides high-density mapping of AID-associated loci for large cohorts at reduced costs. The Immunochip is based on an Illumina Infinium array platform containing 196,524 variants across 186 known autoimmunity risk loci.¹⁴ The variants included in the Immunochip encompass all variants that have been previously described for white European populations (SNPdb, 1000 Genomes Project February 2010 release, and other available sequencing projects). Also, the Immunochip design includes several rare variants considered to have significant functional effects and that might have been previously overlooked.^{14,15}

This approach has recently shown encouraging results in different AIDs, such as celiac disease (CeD [MIM 212750]), rheumatoid arthritis (RA [MIM 180300]), autoimmune thyroid disease (ATD [MIM 275000 and 140300]), psoriasis (PS [MIM 177900]), primary biliary cirrhosis (PBC [MIM 109720]), juvenile idiopathic arthritis (JIA [MIM 604302]), primary sclerosing cholangitis (PSC [MIM 613806]), narcolepsy (MIM 161400), ankylosing spondylitis (MIM 106300), atopic dermatitis (MIM 603165), and Takayasu arteritis (MIM 207600).^{16–27} These studies resulted in the identification of susceptibility genes and the narrowing of the associations in previously reported risk loci. Moreover, these reports increased the number of shared genetic markers between the different disorders, further supporting the common genetic component of autoimmunity.

Taking the above into consideration, the goal of this study was to explore SSc risk loci shared with other autoimmune diseases included on the Immunochip and to fine map these areas, which comprised many, but not all, previously associated SSc loci.

Subjects and Methods

Case Definition

SSc cases were defined on the basis of the 1980 preliminary classification criteria of the American Rheumatism Association (now the American College of Rheumatology)²⁸ or the presence of at least three out of five CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia) features typical of SSc. The designation of lcSSc or dcSSc was determined according to the method of Leroy.² The SSc-specific autoantibodies, ACA and ATA, were determined by standard means as previously described.²⁹

Table 1. Main Clinical Features of the Studied Cohorts

Sample Population	Total After QC	Status and Gender				SSc Subgroups			
		Cases	% Female Cases	Controls	% Female Controls	lcSSc	dcSSc	ACA ⁺	ATA ⁺
US discovery	3,697	956	89%	2,741	65%	594	327	281	155
Spain discovery	1,602	877	89%	725	66%	533	247	390	192
US and Canada replication	1,845	927	86%	918	53%	584	339	279	163
Spain replication	1,237	449	89%	788	56%	238	108	160	85
Germany	1,110	694	83%	416	54%	345	240	237	179
Netherlands	1,470	385	71%	1,085	42%	250	117	90	99
Italy	1,918	648	92%	1,270	81%	436	156	301	213
Sweden	587	229	77%	358	75%	163	66	67	39
UK	1,785	685	84%	1,100	87%	491	191	253	118
Meta-analysis	15,251	5,850	86%	9,401	65%	3,634	1,791	2,058	1,243

Note: 62.17% of cases were lcSSc, and 30.53% were dcSSc (7.3% were not designated); 35.18% of cases were ACA⁺, and 58.85% were ACA⁻ (5.97% were not designated); 21.26% of cases were ATA⁺, and 72.15% were ATA⁻ (6.59% were not designated); and 36.70% of cases were ACA⁺ ATA⁻ (6.86% were not designated). Abbreviations are as follows: ACA⁺, anticentromere-antibody-positive cases; ATA⁺, antitopoisomerase-antibody-positive cases; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; and SSc, systemic sclerosis cases.

Sample Population

After quality-control (QC) measures were applied, the combined data set consisted of 5,850 SSc cases and 9,401 unrelated healthy controls. Table 1 shows the sample populations by sex, origin, and SSc subtype. The discovery cohort consisted of 1,833 SSc cases and 3,466 controls of white European ancestry from the US and Spain. The validation cohort was drawn from an independent group of cases and controls of similar ancestry from North America (US and Canada), Spain, Germany, the Netherlands, Italy, Sweden, and the UK (4,017 SSc cases and 5,935 controls). The populations included in this study partially overlapped with those in previously published SSc GWASs.^{7,9} This study was approved by the local ethics committees of all the centers that recruited the participating individuals, and all participating individuals gave written informed consent.

The discovery population's power to detect an association with an odds ratio (OR) = 1.5 under an additive model at the 5×10^{-6} significance level was 100% (minor allele frequency [MAF] = 0.2), 96% (MAF = 0.1), 56% (MAF = 0.05), and 1% (MAF = 0.01). The overall meta-analysis had a statistical power of 100% (MAF = 0.05) and 18% (MAF = 0.01) with an OR = 1.5 but reached a power of 99% with an OR = 2.00 (the remaining parameters were maintained as previously mentioned). Power calculations were performed as implemented in Power Calculator for Genome-wide Analyses.³⁰

Genotyping

The genotyping for the Immunochip custom array of the discovery cohorts was performed in accordance with Illumina protocols in two centers: the Feinstein Institute for Medical Research (Manhasset) and the Translational Research Institute at the University of Queensland Diamantina Institute (Brisbane). The US and Spanish control cohorts have already been included in previous Immunochip studies.^{17,26}

The genotyping of the replication cohorts was performed with either (1) the TaqMan SNP genotyping technology in the Applied

Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer's suggestions or (2) the Immunochip platform (the controls sets overlapped with those from previous Immunochip reports).^{16–20,22–24,26}

Genotype Calling and QC

Genotype calling on the discovery cohort was performed with the Immunochip platform according to the manufacturer's instructions with the use of the Illumina iScan System and the Genotyping Module (v.1.8.4) of the GenomeStudio Data Analysis software. Stringent QC parameters were applied in all data sets: (1) individuals who generated genotypes at <90% loci were not considered in the analysis, (2) markers with call rates $\leq 90\%$ were excluded, and (3) markers with allele distributions strongly deviating from Hardy-Weinberg equilibrium (HEW) in controls ($p < 1 \times 10^{-5}$) were discarded. After QC measures were applied, 126,270 markers passing QC (101,692 of them showed a MAF > 0.001) were included in the analysis of the discovery cohort, resulting in a genotyping rate of 99.8%. A total of 1,886 SSc cases (895 Spanish and 981 US cases) and 4,629 unaffected controls (781 Spanish and 3,848 US individuals) passed these genotyping QCs.

Individuals in the discovery phase were also excluded on the basis of inferred ethnicity by principal-component analysis (PCA) as implemented in SNP & Variation Suite v.7 (Golden Helix). The first three principal components for each individual were plotted, and those individuals deviating from the cluster centroid were discarded for further analysis. Considering the different reported ancestries in the US control set, we included HapMap Phase 2 samples as reference populations in an initial PCA. Then, we discarded those individuals who deviated more than 6 SDs from the European ancestry cluster centroid (Figure S1, available online). In addition, individuals who deviated more than 4 SDs from the centroid of their cohort were excluded from the analysis. Moreover, to identify duplicate pairs or highly related individuals among data sets, we performed pairwise comparisons by using the genome function in PLINK³¹ and HapMap2 populations as

references (which required Pi-HAT of ≥ 0.5), and we removed one sample from each pair. By these means, 43 SSc cases (18 Spanish individuals and 25 US individuals) and 1,163 controls (56 Spanish and 1,107 US individuals) were not included in the analyzed discovery cohort.

To calculate the genomic-inflation factor (λ) while overcoming the skewed nature of the SNP selection process in the Immuno-chip design, we used a set of 3,120 “null” SNPs not associated with autoimmune diseases (originated by J.C. Barrett and provided by John Bowes, personal communications).^{19,22,23,25,26} The SNPs that did not pass QC or showed an association p value above the 90th percentile in the SSc meta-analysis were discarded. The λ for the discovery sets was estimated at 1.07 (US) and 1.085 (Spain). It has been demonstrated that λ increases with sample size.³² Therefore, it is informative to calculate a rescaled λ for an equivalent study of 1,000 cases and 1,000 controls ($\lambda_{1,000}$).³² In this report, $\lambda_{1,000}$ was estimated at 1.049 (US) and 1.098 (Spain). In the case of the discovery-phase meta-analysis, $\lambda = 1.065$ and $\lambda_{1,000} = 1.02$ (including the associations in the 90th percentile, $\lambda = 1.209$ and $\lambda_{1,000} = 1.064$). All loci showing one or more independent genome-wide-significant associations in the discovery phase remained significantly associated after correction for λ . However, all the identified loci (showing either genome-wide or suggestive significance, Table S1) underwent a replication step.

Imputation

We performed SNP genotype imputation of the identified SSc susceptibility loci (5 Mb regions centered in the lead SNP were considered). We included only discovery-phase genotyped SNPs that passed QC in the imputation process. Genotypes were imputed as implemented in IMPUTE2 with the use of the 1000 Genomes Phase 3 integrated reference panel.^{33,34} We assessed imputed SNP quality by establishing a probability threshold for merging genotypes at 0.9. Moreover, after imputation, stringent QC was applied: (1) individuals who generated genotypes at <90% loci were not considered in the analysis, (2) markers with call rates $\leq 95\%$ were excluded, (3) markers with allele distributions deviated from HWE in controls ($p < 1 \times 10^{-3}$) were discarded, and (4) variants with $\text{MAF} < 0.01$ were not included in the analyses. A total of 390 additional variants were imputed in *ATG5* (autophagy-related 5 [MIM 604261]), 541 in the *SCHIP1* (schwannomin-interacting protein 1 [MIM 612008])–*IL12A* (interleukin 12A [MIM 161560]) locus, and 628 in *DNASE1L3* (deoxyribonuclease 1-like 3 [MIM 602244]).

Imputation of the HLA Region

The 5,955 SNPs obtained in the MHC after all QCs were used for the imputation process. We used as a reference panel for the imputation (1) 2,767 European-descent individuals³⁵ with four-digit typing of human leukocyte antigen (HLA) class I and II molecules and (2) the genotypes of more than 7,500 common SNPs and indel polymorphisms across the extended MHC (xMHC).³⁶ The imputation process was performed with Beagle software³⁷ according to a previously described method.³⁸ Imputed data in the xMHC for SNPs, amino acids, or the HLA four-digit code were filtered as follows: (1) variants with a success call rate below 95%, (2) variants with a MAF below 1%, and (3) all individuals with a SNP success call rate below 95% were excluded. After these filters, a total of 7,261 SNPs remained. Also, through the imputation process, a total of 449 polymorphic amino acid positions were obtained. At last, the alleles of the class I MHC (*HLA-A* [MIM 142800], *HLA-B*

[MIM 142830], and *HLA-C* [MIM 142840]) and class II MHC (*HLA-DPA1* [MIM 142880], *HLA-DPB1* [MIM 142858], *HLA-DQA1* [MIM 146880], *HLA-DQB1* [MIM 604305], and *HLA-DRB1* [MIM 142857]) were obtained. In order to assess the accuracy of the imputation, we used partial data from previously genotyped HLA alleles from 490 individuals from the US and 466 individuals from Spain for *HLA-A*, *HLA-B*, *HLA-C*, *HLA-DPA1*, *HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*, and *HLA-DRB1* at four digits, which were partially included in previous reports.^{39,40} This resulted in an accuracy of 93.51% in the US cohort and 91.70% in the Spanish cohort in the four-digit comparison (Table S2).

Statistical Analysis

The association statistics for the discovery cohorts were calculated separately in each data set via logistic regression including sex as a covariate. Then, meta-analysis combining ORs and SEs of both cohorts was performed via the inverse-variance method under the assumption of a fixed effect as implemented in PLINK v.1.07.³¹ Heterogeneity across the data sets was assessed with Cochran's Q test, and those loci showing significant heterogeneity ($Q < 0.05$) were not considered for the validation step.

The validation SNPs were chosen for the validation phase if they (1) had a meta-analysis $p < 5 \times 10^{-6}$, (2) had a nominal association in both cohorts at $p < 0.05$, (3) had consistent OR directions in both cohorts, (4) were not located in a gene previously identified as SSc associated, (5) and mapped outside the HLA region (considered the loci located in chr6: 20,000,000–40,000,000 bp).

The possibility of different independent signals in the same locus was evaluated with a stepwise logistic regression as described in Eyre et al.¹⁷ In brief, the most associated variant in the region was considered a covariate and the association analyses were calculated for the remaining variants. Independent signals should have met the following criteria: (1) $p < 5 \times 10^{-6}$, (2) no high correlation with the lead SNP ($r^2 < 0.6$), and (3) no substantial difference between the conditioned and the unconditioned p values ($p < 5 \times 10^{-4}$).

The combined analysis of the discovery populations with the replication cohorts was performed via the inverse-variance method under a fixed-effects model on the basis of population-specific logistic regression results. Only variants showing a genome-wide-significant association ($p < 5 \times 10^{-8}$), either in the whole disease or in any of the subgroup analyses, were considered confirmed associations with SSc susceptibility.

For the analyses of the HLA region, we used logistic regression to test for allelic association between the imputed variant and disease status. Individual amino acid positions were tested with a model including all the possible amino acid residues. Statistical significance was established by comparison of the deviance model to the null model.³⁸ For the conditional analyses, we assumed that the null model comprised the previously associated variants in a stepwise manner until no genome-wide-significant associations remained in the HLA region.

Functional Prioritization of Noncoding Variants

In addition to analyzing coding variants (functional prediction was based on PolyPhen-2),⁴¹ we propose most likely functional variants that might explain the associations on the basis of noncoding element data. We used the Regulome, Genevar, and Blood eQTL browser resources to explore the evidence of transcription factor binding sites, DNase hypersensitivity sites, and expression quantitative trait loci (eQTLs) in the publicly available

ENCODE database and additional eQTL databases.^{42–44} Molecular graphics and analyses of DNASE1L3, HLA-DPAβ1, HLA-DQα1, and HLA-DRβ1 structure were performed with the UCSF Chimera package.⁴⁵

Results

The HLA region showed the most significant associations in the discovery phase, and three already reported non-HLA SSc risk factors (*STAT4*, *PXK*, and the *TNPO3* [MIM 610032]-*IRF5* locus)^{7,13,46–54} showed genome-wide-significant associations with the disease (Table S3). No independent signals as previously defined were observed in these loci. Additionally, we calculated blocks of high linkage disequilibrium (LD) for the leading signals (defined as those SNPs showing $r^2 > 0.9$ with the lead variant according to the 1000 Genomes Project CEU [Utah residents with ancestry from northern and western Europe from the CEPH collection] population) in these loci.³⁴ By these means, we established an association region comprising 35 kb for the leading variant in *STAT4*, 126 kb in the case of the *TNPO3-IRF5* locus, and 91 kb in *PXK*. Because of their proximity, we tested for possible dependence between the signals in *PXK* and *DNASE1L3*. Interestingly, both the genome-wide association observed in our data in *PXK* (rs4681851) and the reported association with SSc in *PXK* (rs2176082)¹³ were dependent on the association located in *DNASE1L3* ($p_{\text{cond}} \text{ rs4681851} = 0.19$, $p_{\text{cond}} \text{ rs35677470} = 3.71 \times 10^{-8}$; $p_{\text{cond}} \text{ rs2176082} = 0.12$, $p_{\text{cond}} \text{ rs35677470} = 6.41 \times 10^{-11}$). In addition, several previously described SSc susceptibility factors showed significant associations with SSc or the different subphenotypes of the disease and were confirmed in our analysis (Table S3). In this study, we performed a comprehensive analysis of the associations in the HLA region and focused on the associations previously undescribed in non-HLA loci.

Associations between the HLA Region and Systemic Sclerosis

We also conducted the imputation of the HLA region (chr6: 28,000,000–34,000,000 bp) in our data by using a large reference panel as previously described. Resulting from the imputation, we obtained 7,261 SNPs, 449 polymorphic amino acid positions, and 298 four-digit HLA alleles for both class I and class II molecules. Compared to HLA typing, the imputation had an overall accuracy of 93.08%.

Taking into account the serologically restricted HLA association in previous reports,⁹ we conducted different analyses to compare total SSc, ACA⁺, and ATA⁺ between cases and controls in this locus. The overall results from the analysis specific to the HLA region can be observed in Figure 1 and Table 2. After stepwise conditional multiple logistic regression analysis in the aforementioned groups, we obtained a model composed of six polymorphic amino acid positions (two and four of which were associated with ACA and ATA, respectively, Table 2) and seven SNPs (five

and two of which were associated with SSc and ACA, respectively, Table 2), which successfully conditioned all observed associations in the HLA region in either SSc or any of its serological subphenotypes (Figure 1). Consequently, the identified models conditioned the known SSc-related HLA four-digit alleles (Table S4).³⁹

Loci Revealed in the Immunochip Analyses

Eight SNPs were selected as putative SSc risk factors (Table S1). We genotyped these variants in a large multicenter replication cohort of European ancestry in order to confirm the initial associations in the discovery phase. One SNP failed to genotype in the North American replication cohort, as indicated in Table S5. As illustrated in Figure 2 and Table 3, the meta-analysis of the European and North American discovery and replication cohorts identified three non-HLA loci to be associated with overall SSc ($p < 5 \times 10^{-8}$, see Table S5 for results of all selected SNPs in all groups). The associated variants included a missense SNP (rs35677470) in *DNASE1L3* at 3p14 (resulting in a cysteine substitution for arginine in the gene product, Figure S2), a SNP (rs77583790) in the intergenic region between *SCHIP1* and *IL12A* at 3q25, and a SNP (rs9373839) intronic to *ATG5* at 6q21. In addition, a SNP (rs7130875) in the intergenic region between *TREH* (trehalase [MIM 275360]) and *DDX6* (DEAD-box RNA helicase 6 [MIM 600326]) at 11q23 showed suggestive association ($p = 1.29 \times 10^{-7}$, OR = 1.17).¹⁷

The association signals for *DNASE1L3* and *SCHIP1-IL12A* were also significant in the lcSSc and ACA⁺ subgroups. Moreover, in the case of *DNASE1L3*, the signal was strongly related to ACA⁺ SSc ($p = 4.25 \times 10^{-31}$, OR = 2.03), and the most significant association in the *SCHIP1-IL12A* region was found with lcSSc ($p = 1.53 \times 10^{-11}$, OR = 2.81) (Table 3). Of note, there was no previously unreported genome-wide-significant association with either the dcSSc or the ATA⁺ subset of disease in this cohort. It is worth mentioning that the risk effects for the replicated polymorphisms were maintained in all the analyzed populations (Figure 2 and Figures S3 and S4).

In order to further dissect the associated loci, we imputed the associated regions by using the 1000 Genomes Project reference panel, as described previously. Imputation resulted in a significant increase in the number of analyzed variants, but only slight differences between the top genotyped SNPs and the top imputed SNPs were observed. Considering the dense coverage of the fine-mapped loci in the Immunochip, this low gain of information from imputation was concordant with previous Immunochip reports.^{22,26} As expected, the linked variants in the regions surrounding the association peaks showed concordant associations (Figure 3). However, no additional independent signals, neither SNPs nor haplotypes, showed up after conditioning on the lead variant (Figure 3).

In the case of *DNASE1L3*, the lead variant (rs35677470) encodes a probably damaging nonsynonymous change, as mentioned above. No additional variant in high LD

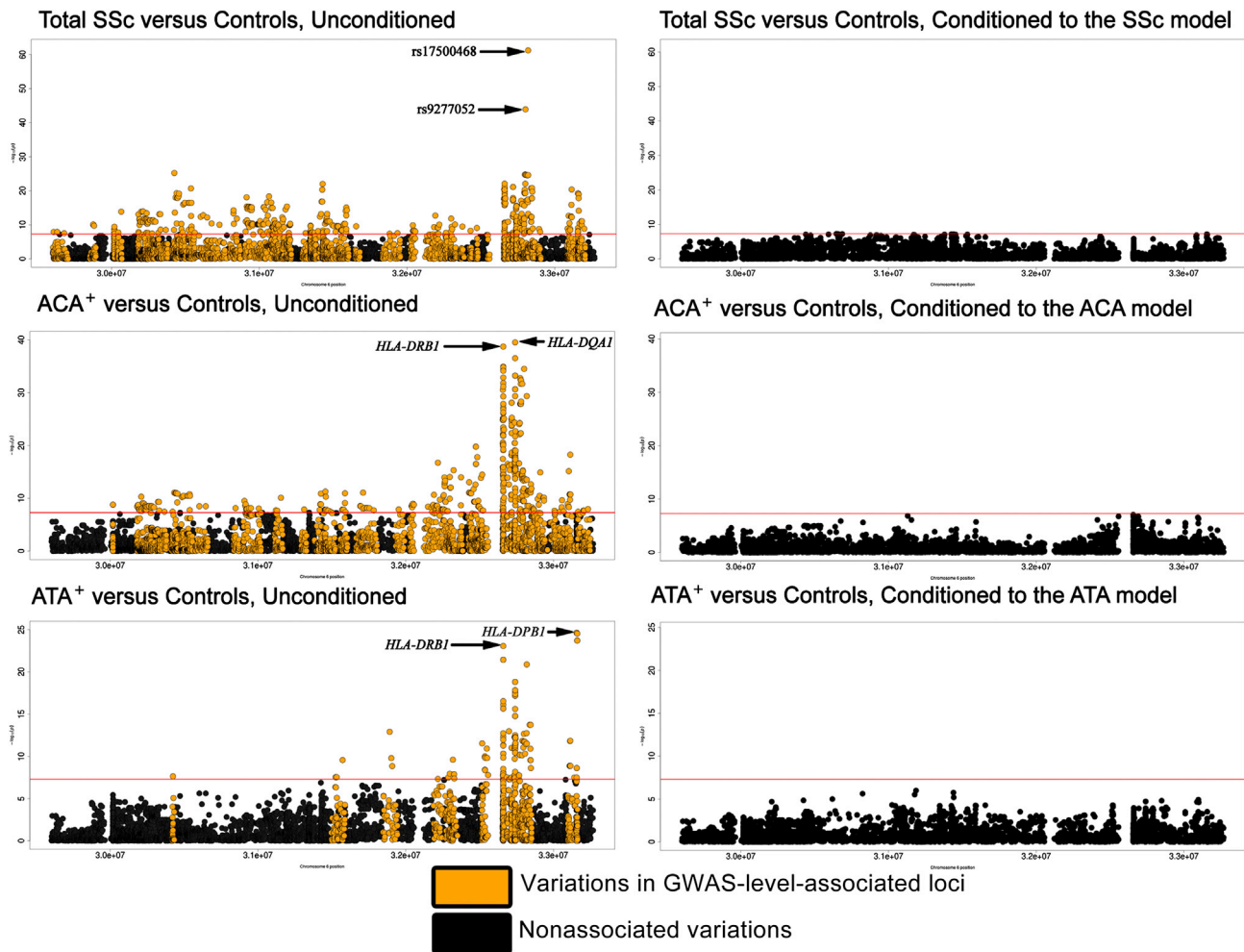


Figure 1. HLA Region: 28,000,000–36,000,000 bp

Manhattan plots in which each dot represents the p value of a variant ($-\log_{10}(\text{p value})$) on the vertical axis and the position in chromosome 6 on the horizontal axis. The ACA model comprised HLA-DRB1 amino acid 13, HLA-DQA1 amino acid 69, and SNPs rs12528892 and rs6933319. The ATA model comprised HLA-DRB1 amino acids 67 and 86 and HLA-DPB1 amino acids 76 and 96. The SSc model included the previously described ACA and ATA models and SNPs rs17500468, rs9277052, rs2442719, and rs4713605.

with this SNP ($r^2 > 0.9$) was located in a coding region or has been associated with eQTLs. Therefore, we suggest that the lead variant itself might be the most suitable causal candidate.

No variants linked with the *ATG5* leading variant have protein-coding consequences or have been associated with eQTLs. Four variants located in the fifth *ATG5* intron (rs62422881, rs3804333, rs1885450, and rs698029) were located in transcription factor binding sites and DNase peaks (Regulome scores = 2a–4). This region showed suggestive evidence of binding of different proteins with roles in the immune system (e.g., PRDM1, EP300, or CEBPB). In addition, rs7763652, a variant in the *ATG5* promoter region, showed minimal evidence of affecting the binding of CTCF (Regulome score = 4). Further research might shed light on the functional relevance of this region.

Variants in the *SCHIP1-IL12A* locus showed only minimal evidence of binding. Nevertheless, this lack of functional information might be due to the low MAF.

Discussion

In the present study, we were able to further dissect the long-known association between the HLA region and SSc. We narrowed the genetic factors in this region down to a model of six amino acids and seven SNPs, all in the ACA⁺ and ATA⁺ subgroups (except for rs17500468, rs9277052, rs2442719, rs4713605, and rs443623, which presented their association in the overall disease). We further checked all associated SNPs for any functional explanation of their association. Among other methods, we checked whether any of the SNPs were in a putative eQTL according to Westra et al.⁴⁴ SNPs rs17500468 and rs2442719 (associated with SSc) were in an eQTL affecting the expression of the non-HLA gene *TAP2* (MIM 170261; $p = 4.90 \times 10^{-10}$ and 4.23×10^{-8} , respectively), rs9277052 (associated with SSc) was in an eQTL affecting the expression of *HLA-DPB1* ($p = 2.86 \times 10^{-69}$), SNP rs4713605 was in an eQTL affecting the expression of

Table 2. All Independent Associations Found between the HLA Region and SSc or Any of Its Subphenotypes

SSc Subgroup	Type	Gene	Variation	AA or Nucleotide	Frequency in Cases	Frequency in Controls	p Value ^a	OR ^b	95% CI ^b
ACA ⁺	AA	<i>HLA-DRB1</i>	AA 13 ^c	Tyr	0.048	0.139	1.79×10^{-39}	0.295	0.22–0.38
				Phe	0.232	0.136		1.817	1.56–2.11
ACA ⁺	AA	<i>HLA-DQA1</i>	AA 69 ^c	Leu	0.484	0.581	4.46×10^{-23}	0.657	0.58–0.75
				T	0.076	0.020		3.788	2.85–5.02
ACA ⁺	SNP	<i>TAP2</i>	rs12528892 ^d	T	0.064	0.011	2.74×10^{-11}	9.137	6.49–12.85
ACA ⁺	SNP	<i>HLA-DOA</i>	rs6933319	T	0.030	0.056	7.13×10^{-16}	0.304	0.21–0.42
SSc	SNP	<i>TAP2</i>	rs17500468 ^d	G	0.186	0.082	5.87×10^{-62}	2.868	2.52–3.25
SSc	SNP	<i>HLA-DPB1</i>	rs9277052 ^d	C	0.275	0.182	4.08×10^{-21}	1.572	1.43–1.72
SSc	SNP	<i>HLA-B</i>	rs2442719 ^d	G	0.491	0.410	9.55×10^{-23}	1.512	1.39–1.64
SSc	SNP	<i>HLA-DPB1</i>	rs4713605 ^d	A	0.381	0.435	2.16×10^{-13}	0.726	0.66–0.79
SSc	SNP	<i>HLA-DOA</i>	rs443623	A	0.227	0.304	3.49×10^{-9}	0.770	0.70–0.84
ATA ⁺	AA	<i>HLA-DRB1</i>	AA 67	Leu	0.227	0.388	3.55×10^{-22}	0.461	0.38–0.55
				Phe	0.311	0.173		2.158	1.80–2.58
ATA ⁺	AA	<i>HLA-DPB1</i>	AA 96	no Lys or Arg ^c	0.037	0.001	3.21×10^{-23}	75.230	28.09–201.50
ATA ⁺	AA	<i>HLA-DRB1</i>	AA 86 ^c	Val	0.573	0.476	1.77×10^{-5}	1.423	1.21–1.67
ATA ⁺	AA	<i>HLA-DPB1</i>	AA 76 ^c	Ile	0.074	0.028	3.05×10^{-8}	2.577	1.85–3.59

Abbreviations are as follows: AA, amino acid; ACA⁺, anticentromere-antibody-positive cases; ATA⁺, antitopoisomerase-antibody-positive cases; CI, confidence interval; OR, odds ratio; and SSc, systemic sclerosis cases.

^aLogistic regression omnibus test for variations with more than two alleles.

^bORs and CIs for each of the associated alleles.

^cAmino acid part of the binding pocket of the molecule.

^dLocated in a putative eQTL with a p value of at least 5×10^{-8} according to Westra et al.

^eThe absence of a lysine or an arginine in this position is the associated variant, which is very infrequent in healthy individuals of European ancestry.

HLA-DPB1 ($p = 2.26 \times 10^{-81}$), and rs12528892 was also in an eQTL affecting the expression of *TAP2* ($p = 8.53 \times 10^{-68}$). Finally, SNPs rs443623 and rs6933319 (associated with SSc and ACA production, respectively) were not predicted to be in any eQTL or a likely transcription factor binding site; the closest gene was *HLA-DOA* (MIM 142930), whose product forms an heterodimer with *HLA-DOβ* to assemble the HLA-DO molecule, found in lysosomes in B cells regulating HLA-DM-mediated peptide loading on MHC class II molecules.⁵⁵ Regarding the other described genes affected by eQTL SNPs, *HLA-DPB1* encodes the β chain of the HLA-DP MHC receptor complex, whereas *TAP2* is involved in antigen presentation.⁵⁶

Regarding the amino-acid-associated positions, we found that the associated amino acid at position 13 in *HLA-DRβ1* was part of the binding pocket of this molecule with epitope,⁵⁷ which was also the case with amino acid 69 in *HLA-DQα1*,⁵⁸ both of these amino acids are associated with ACA production (Figure S5). Amino acids 86 and 76 in *HLA-DRβ1* and *HLA-DPβ1*, respectively (both associated with ATA production), were also part of the binding pocket of their respective molecules.^{57,59} Conversely, amino acids 67 and 96 in *HLA-DRβ1* and *HLA-DPβ1*, respectively (associated with ATA production, Table 2), were not part of the binding pocket but could affect the 3D structure of the MHC. Hence, the complexity and heterogeneity of

the associations between the HLA region and SSc reflect the complex and heterogeneous nature of this disorder.

Three non-HLA variants associated with SSc susceptibility were clearly identified in this report, increasing the number of loci that have been associated with SSc and providing additional insight into SSc pathogenesis. Moreover, the nonsynonymous risk variant in *DNASE1L3* showed a highly significant association with the ACA⁺ group considering the known non-HLA genetic associations with SSc.^{4–6}

DNASE1L3 codes for deoxyribonuclease 1-like 3, a member of the human DNase 1 family, which is expressed primarily in the liver and spleen and secreted by macrophages.⁶⁰ This protein plays a role in DNA fragmentation during apoptosis and in the generation of resected double-strand breaks in immunoglobulin-encoding genes.^{61–63} Moreover, it has been implicated in susceptibility to SLE and RA.^{17,64} The nonsynonymous rs35677470 SNP in *DNASE1L3* exon 8 results in a Arg-to-Cys substitution at amino acid position 206 of the protein (p.Arg206Cys). This amino acid change results in the loss of a hydrogen bond in the protein (Figure S2), and in vitro studies have indicated that the protein constructed with this substitution lacks DNase activity; this evidence suggests a potential role in autoimmunity for this SNP.⁶⁵ Moreover, the observed genome-wide associations in *PXK*

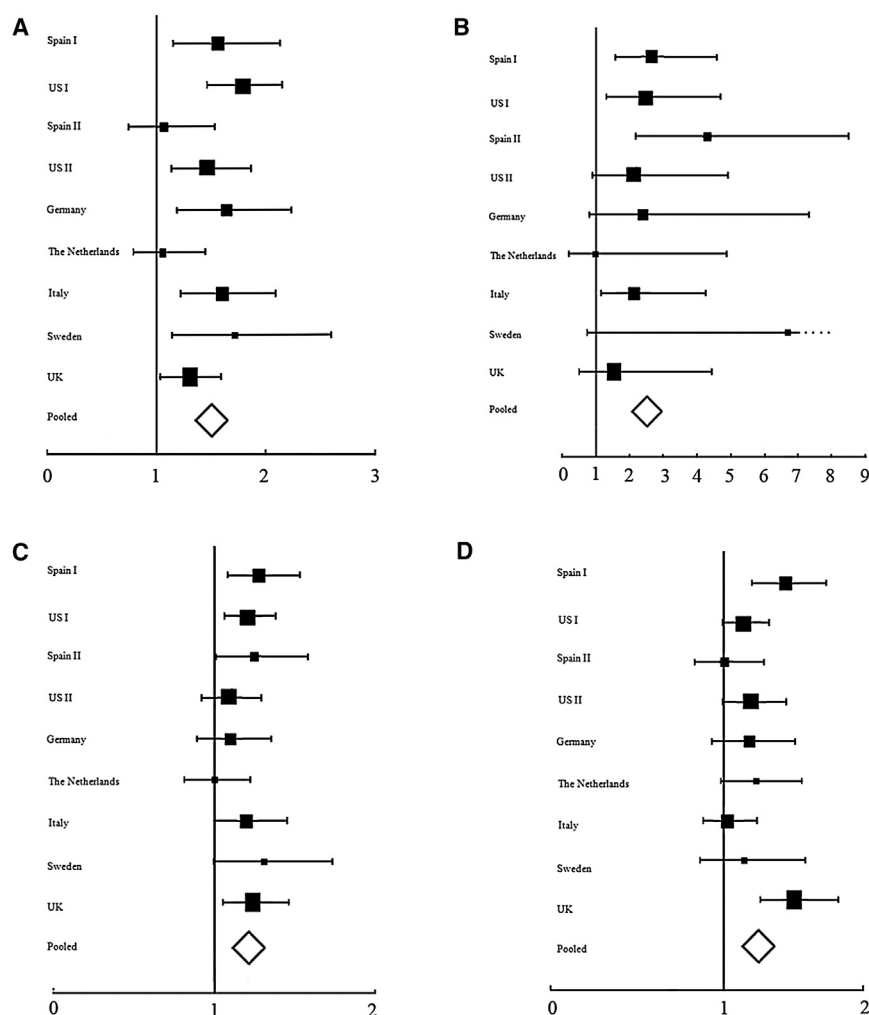


Figure 2. Forest Plots Showing the Population-Specific and Pooled Analyses of the Variants at Genome-wide or Suggestive Significance in the SSc versus Control Analysis

(A) rs35677470 in *DNASE1L3*.

(B) rs77583790 in the *SCHIP1-IL12A* locus.

(C) rs9373839 in *ATG5*.

(D) rs7130875 in the *TREH-DDX6* locus.

gene beyond the currently reported association in familial SLE cases.⁶³

As noted in the [Subjects and Methods](#), our study reached a high statistical power to detect associations with large effects despite low frequency of the minor allele. In fact, a rare variant in the intergenic region between *SCHIP1* and *IL12A* has been firmly associated with SSc, particularly the lcSSc subset. The same region was also identified in the Immunochip analysis in CeD.¹⁶ Trynka et al. identified three different signals (rs76830965, rs1353248, and rs2561288) in this region.¹⁶ However, these variants were not associated in our data ($p > 0.05$), and our lead SNP (rs77583790) was not linked to the previously mentioned polymorphisms. On the contrary, rs80014155, one of the four signals observed in PBC,²⁰ showed $r^2 = 0.97$ with our lead SNP. Therefore, both sig-

were in modest LD with and dependent on the *DNASE1L3* variant. Thus, we hypothesize that because of its functional implications, the rs35677470 polymorphism is likely to be the causal variant for the reported associations with SSc in the region. Considering that this variant is exclusive to populations with European ancestry and has a relatively low frequency and only two proxy SNPs ($r^2 > 0.8$), it is not surprising that it remained undiscovered and that fine mapping of the region was required for identifying this highly significant association. Interestingly, vascular injury and immune deregulation, which are both SSc hallmarks, are related to cell damage and deregulated apoptosis.⁶⁶ Therefore, the association between SSc (especially ACA⁺ SSc) and the rs35677470 loss-of-function variant in *DNASE1L3* might provide a link between a defective apoptotic DNA breakdown and the production of ACA.

Utilizing the Immunochip fine-mapping platform, Eyre et al. confirmed the same SNP as the SNP most highly associated with RA in the region,¹⁷ which supports the role of this gene and this specific variant as a common autoimmunity risk factor. Direct genotyping of this polymorphism in nonfamilial SLE might shed light on the relevance of this

nals were equivalent. The location of the SSc signal in the region upstream of *IL12A* might point to an effect of this SNP (or a linked variant) in the regulation of *IL12A* expression. Moreover, different loci in the IL12 pathway have been associated with SSc (i.e., *IL12RB2* and *STAT4*), and altered levels of IL12 have been detected in SSc-affected individuals,^{7,11,52,54,67} supporting a relevant role for this interleukin pathway in the disease. Nevertheless, because of the lack of functional data on this variant, its low frequency, and the high level of LD in this region, an unknown role of *SCHIP1* or other surrounding loci should not be discarded.

Regarding the association located in *ATG5*, the *ATG5*-encoded protein forms a complex with ATG12 and assists in autophagosomal elongation.⁶⁸ Remarkably, this signal at the genome-wide level links SSc with autophagy. Autophagy is a degradation pathway that mediates pathogen clearance and allows cells to degrade unwanted cytoplasmic material and to recycle nutrients.⁶⁸ It plays a key role in both innate and adaptive immune system development and responses and has been associated with a number of AIDs.⁶⁸ Along this line, several GWASs have found associations between the autophagy-related gene *ATG5* and SLE susceptibility, as well as risk of childhood and

Table 3. Non-HLA Loci Associated with SSc and Its Subsets and Identified through Immunochip Analysis									
Locus	SNP	Chromosomal Region	Minor Allele	LD Region $r^2 > 0.9$	Region size		Comments	Phenotype	MAF Cases/Controls
					(kb)				p Value
DNASE1L3	rs35677470	3p14	A	rs139325797, rs35677470	2		missense Arg > Cys	SSc	0.100/0.062
								lcSSc	1.28 × 10 ⁻²⁰
SCHIP1-IL12A	rs77583790	3q25	A	rs183665802, rs186702062, rs184541632, rs185514275, rs80014155, rs77583790, rs114591798, rs73154557, rs187938609, rs200875363, rs73155915, rs73155982	1,641		intergenic	ACA ⁺	0.133/0.062
								SSc	1.22 × 10 ⁻¹¹
								lcSSc	1.53 × 10⁻¹¹
ATG5	rs9373839	6q25	G	rs34599047, rs34582442, rs1322178, rs9386514, rs62422862, rs34936565, rs9398073, rs9399978, rs9373839, rs9372120, rs2299864, rs9386516, rs62422878, rs77791277, rs3827644, rs3804329, rs34843857, rs62422881, rs9398075, rs11752888, rs10484577, rs62422886, rs3804333, rs9373842, rs9372121, rs151290510, rs9373843, rs9398078, rs66469051, rs9373846, rs7763652	157		intronic	ACA ⁺	2.40 × 10 ⁻⁸
								SSc	3.75 × 10 ⁻⁸
									1.19

The phenotypes with the most significant associations are shown in bold. Abbreviations are as follows: ACA⁺, antitopoisomerase-antibody-positive cases; ATA⁺, antitopoisomerase-antibody-positive cases; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; LD, linkage disequilibrium; MAF, minor allele frequency; OR, odds ratio; p value, p value of the meta-analysis using the inverse variance method under a fixed effects model; and SSc, systemic sclerosis cases.

adult asthma and decline in lung function.⁶⁸ However, recently published fine-mapping studies in RA, CeD, PS, ATD, PBC, JIA, and narcolepsy did not find associations with this gene region.^{16–24} Thus, this locus appears to be restricted to some, but not all, AIDs. In any case, the intronic location and the protein binding data of the variants in the *ATG5* region suggest that the functional meaning of these variants might be complex and involve distant genes (even *PRDM1* [MIM 603423], which maps downstream of *ATG5* and has been associated with different autoimmune diseases^{69–71}). Moreover, there is evidence of a possible binding of CTCF in the rs7763652 region. This protein is involved in chromatin architecture and DNA-loop formation,⁷² which brings up the possibility of a complex DNA structure in the region. Therefore, we consider that future research will be needed for determining the functional implications of this signal.

We also identified a suggestive association in the 11q23 intergenic region between *TREH* and *DDX6*, which has been shown to be a shared susceptibility region among several AIDs. Nevertheless, the different lead SNPs in *DDX6* in the Immunochip analysis of RA (rs4938573),¹⁷ CeD (rs10892258),¹⁶ and PBC (rs80065107)²⁰ were not linked with this SSc-suggestive variant. The gene product of *DDX6* (an RNA helicase that is important for efficient miRNA-induced gene silencing) has been shown to regulate vascular endothelial growth factor under hypoxic conditions, which might provide a clue to the vasculopathy and fibrosis that characterize SSc.⁷³

The association of these loci provides genetic evidence of the possible role of defects in DNA elimination during apoptosis, introduces autophagy as a pathogenic mechanism, and reinforces the role of the IL12 pathway in the pathogenic processes that lead to SSc onset and disease progression. Therefore, we consider that the present study, together with previous knowledge about the genetic component of SSc, has contributed to the notion of this disorder as a complex condition in which several biological mechanisms, such as the innate immune response (Toll-like receptor and type I IFN pathways), the adaptive immune response (especially the IL12 pathway), tissue damage, fibrotic processes, and now DNA-clearance mechanisms during apoptosis, interact. However, neither these mechanisms nor the involved genetic loci should be considered independent compartments but rather pleiotropic players in a genetic and phenotypic continuum.

The analysis of genome-wide associations in already known loci resulted in the fine mapping of previously reported signals in *STAT4* and *IRF5*. *STAT4* is a shared susceptibility factor among RA, CeD, PBC, and JIA, but the most associated SNPs in the different conditions vary.^{16,17,20–22} In the case of the *TNPO3-IRF5* locus, different variants have been associated with multiple autoimmune diseases and previous Immunochip analyses have reported different lead SNPs in RA and PBC.^{17,20,21} In our study, the leading signals in these genes were highly linked with the RA-associated variants. The dense mapping of these established SSc

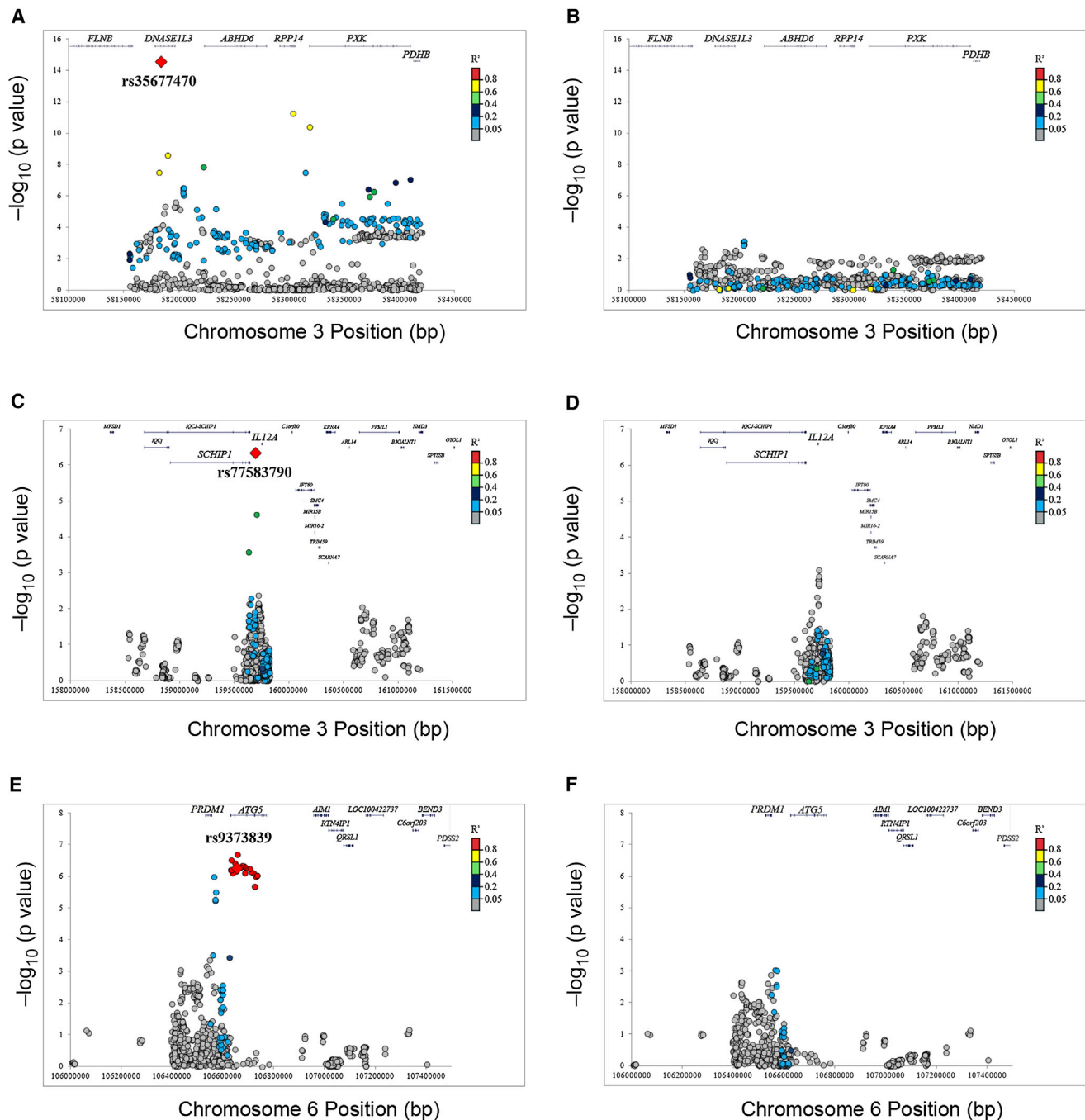


Figure 3. Regional Plots of the Associations Replicated at Genome-wide Significance in SSc Cases or Different Subgroups in the Overall Meta-analysis after Imputation

- (A) *DNASE1L3* associations in the ACA⁺ versus control comparison.
 (B) *DNASE1L3* associations in the ACA⁺ versus control comparison after conditioning on the lead variant (rs35677470).
 (C) *SCHIP1-IL12A* locus associations in the lcSSc versus control comparison.
 (D) *SCHIP1-IL12A* locus associations in the lcSSc versus control comparison after conditioning on the lead variant (rs77583790).
 (E) *ATG5* associations in the dcSSc versus control comparison.
 (F) *ATG5* associations in the dcSSc versus control comparison after conditioning on the lead variant (rs9373839).
 p values correspond to the discovery phase.

susceptibility factors resulted in the confirmation of previous signals and narrowed association regions in these loci (35 kb in *STAT4* and 126 kb in *IRF5*, considering the SNPs showing $r^2 > 0.9$ with the lead variant in the locus according to the 1000 Genomes Project CEU population).³⁴

In the case of *IL12RB2* and *TNIP1*, both loci showed suggestive associations but did not reach genome-wide significance. In previous reports, similar results were observed in the discovery phases in which these loci did not reach genome-wide significance and large replication cohorts

were needed for confirming these associations.^{8,10,11} Therefore, we consider that our data reinforce the evidence of a role for these genes in SSc; however, larger cohorts should be fine mapped for narrowing down the observed signals.

As a result of design limitations, not all the known variants in *CD247* and *CSK* were included in the Immunochip (which was planned prior to the SSc GWAS), and these regions were therefore only covered by a tag SNP strategy. Unfortunately, after QC, the tagging SNPs for the reported associations in these regions were not maintained for further analysis. Thus, the initial signals were not covered, and this study could not address the confirmation of these previous findings.

It is worth mentioning that, as intended in the Immunochip design, our data showed an expected common genetic background between SSc and other AIDs. The genetic similarities between SSc and SLE are well known,^{4,13} but our results reinforce the evidence of overlap with other AIDs, such as RA or PBC. The identification of common autoimmune risk factors is essential for the characterization of different pathogenic mechanisms that contribute to autoimmunity. Moreover, these common pathways might shed light on the origin of coautoimmunity and the preclinical stages of autoimmunity. In addition, relevant information can be inferred from the existence of different signals in the susceptibility regions or the lack of association between certain loci and specific diseases. Therefore, it has been suggested that platforms such as the Immunochip will help to dissect the pathogenic mechanisms underlying multiple disease states and lead to both more sensitive diagnostics and novel therapies.⁷⁴

In summary, we have provided a comprehensive analysis of associations between the HLA region and SSc and its subphenotypes. Moreover, we report associations between *DNASE1L3*, the *SCHIP1-IL12A* locus, and *ATG5* and SSc and a suggestive association between the *TREH-DDX6* locus and SSc. The Immunochip-based interrogation of the analyzed cohorts revealed shared associations with other autoimmune diseases, which was the goal of the Immunochip Consortium, but also identified intriguing differences. Moreover, our data underline the need for direct genotyping of virtually all functional polymorphisms and rare variants in large cohorts for identifying variants that have strong effects in disease susceptibility but that might have been ignored thus far.

Supplemental Data

Supplemental Data include full affiliations for the Spanish Scleroderma Group, five figures, and five tables and can be found with this article online at <http://www.cell.com/AJHG>.

Consortia

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://www.1000genomes.org/>
BEAGLE, <http://faculty.washington.edu/browning/beagle/beagle.html>
Blood eQTL Browser, <http://genenetwork.nl/bloodeqtlbrowser/>
ENCODE, <http://genome.ucsc.edu/ENCODE/>
Genevar (Gene Expression Variation), <http://www.sanger.ac.uk/resources/software/genevar/>
HLA Nomenclature, <http://hla.alleles.org/>
HUGO Gene Nomenclature Committee, <http://www.genenames.org/>
IMPUTE2, http://mathgen.stats.ox.ac.uk/impute/impute_v2.html
LocusZoom, <http://csg.sph.umich.edu/locuszoom/>

NCBI, <http://www.ncbi.nlm.nih.gov/>
 Online Mendelian Inheritance in Man (OMIM), <http://omim.org/>
 PLINK, <http://pngu.mgh.harvard.edu/~purcell/plink/>
 PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
 RegulomeDB, <http://RegulomeDB.org/>
 UCSF Chimera, <http://www.cgl.ucsf.edu/chimera/>

Accession Numbers

The dbGaP accession number for the genotype information of the US cohort presented in this paper is phs000357.v2.p2.

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